Neuregulin/ErbB Signaling Regulates Cardiac Subtype Specification in Differentiating Human Embryonic Stem Cells

Wei-Zhong Zhu, Yiheng Xie, Kara White Moyes, Joseph D. Gold, Bardia Askari, Michael A. Laflamme

Rationale: Human embryonic stem cell–derived cardiomyocytes (hESC-CMs) exhibit either a “working” chamber or a nodal-like phenotype. To generate optimal hESC-CM preparations for eventual clinical application in cell-based therapies, we will need to control their differentiation into these specialized cardiac subtypes.

Objective: To demonstrate intact neuregulin (NRG)-1β/ErbB signaling in hESC-CMs and test the hypothesis that this signaling pathway regulates cardiac subtype abundance in hESC-CM cultures.

Methods and Results: All experiments used hESC-CM cultures generated using our recently reported directed differentiation protocol. To support subsequent action potential phenotyping approaches and provide a higher-throughput method of determining cardiac subtype, we first developed and validated a novel genetic label that identifies nodal-type hESC-CMs. Next, control hESC-CM preparations were compared to those differentiated in the presence of exogenous NRG-1β, an anti-NRG-1β neutralizing antibody, or the ErbB antagonist AG1478. We used 3 independent approaches to determine the ratio of cardiac subtypes in the resultant populations: direct action potential phenotyping under current-clamp, activation of the aforementioned genetic label, and subtype-specific marker expression by RT-PCR. Using all 3 end points, we found that inhibition of NRG-1β/ErbB signaling greatly enhanced the proportion of cells showing the nodal phenotype.

Conclusions: NRG-1β/ErbB signaling regulates the ratio of nodal- to working-type cells in differentiating hESC-CM cultures and presumably functions similarly during early human heart development. We speculate that, by manipulating NRG-1β/ErbB signaling, it will be possible to generate preparations of enriched working-type myocytes for infarct repair, or, conversely, nodal cells for potential use in a biological pacemaker. (Circ Res. 2010;107:776-786.)

Key Words: embryonic stem cell ■ electrophysiology ■ pacemaker ■ neuregulin

A number of groups, including our own, have reported methods for generating large quantities of highly purified cardiomyocytes from human embryonic stem cells (hESCs) and shown that transplantation of these cardiomyocytes can partially remuscularize infarcted rodent hearts and help preserve contractile function.1-3 However, concerns remain that currently available hESC-derived cardiomyocyte (hESC-CM) preparations include myocytes with both nodal and “working” (ie, atrial and ventricular chamber) type action potential (AP) properties.4-6 This electrophysiologic diversity, also reported for cardiomyocytes derived from murine ESCs (mESCs),7-9 induced pluripotent stem cells,10 and resident cardiac stem cells,11 represents both an opportunity and a challenge to the development of stem cell-based therapies. An enriched preparation of nodal cells would be of potential use in the formation of a biological pacemaker. On the other hand, we may want to exclude nodal cells from cardiomyocyte preparations for infarct repair, as their sustained pacemaking activity and unique neurohormonal responsiveness could exacerbate the already elevated risk of arrhythmias.12,13

Our directed cardiac differentiation protocol for hESCs reliably produces populations of high cardiac purity,1 so we reasoned that this culture system was an excellent platform to screen pharmacological interventions for their effect on the relative abundance of nodal and working-type cardiomyocytes. A number of signaling molecules have been implicated in the development of specialized cardiac subtypes, including neuregulin (NRG)-1β,14-17 endothelin,18 retinoic acid,19 and Wnt family ligands.20 Although we are continuing to examine several of these for their effects on cardiac subtype specification in differentiating hESC-CMs, we focused initially on the NRG-1β/ErbB signaling pathway. Neuregulins are members of the epidermal growth factor family, and they signal via the receptor tyrosine kinases ErbB2, ErbB3 and ErbB4 to regulate the proliferation, survival and differentiation of...
multiple cell types, including cardiomyocytes. The functions of NRG-2 to 4 in cardiomyocytes are unknown, but NRG-1β is known to be an important regulator of both cardiac development and postnatal function, in the murine embryonic heart tube, NRG-1β is released by the ventricular endocardium, whereas adjacent cardiomyocytes express the corresponding ErbB2 and ErbB4 receptors. NRG-1, ErbB2-, and ErbB4-knockout mice all show an embryonic lethal phenotype with a failure to undergo expansion and trabeculation of the primitive ventricle.

Consistent with this, NRG-1β has been shown to promote the maturation and proliferation of primary ventricular cardiomyocytes isolated from the developing rodent heart. Moreover, work in murine and zebrafish models has implicated NRG-1β/ErbB signaling as a critical regulator in the development of specialized nodal and conduction structures.

Based on the preceding data, we hypothesized that NRG-1β/ErbB signaling regulates the relative abundance of nodal-to-working-type cardiomyocytes in hESC-CM cultures. To test this hypothesis, we used 3 independent approaches: direct AP phenotyping, activation of a novel subtype-specific genetic reporter, and RT-PCR for a panel of subtype-specific genes. By all 3 end points, inhibition of NRG-1β/ErbB signaling greatly increased the proportion of cells with the nodal phenotype, whereas its activation favored the opposite effect. In addition to providing important insights into the regulation of subtype specification in early human hearts, these findings suggest pharmacological approaches to derive subtype-enriched preparations for use in cell-based therapies.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

In brief, cardiomyocytes were generated from H7 hESCs using our recently reported directed differentiation protocol, which involves serum-free monolayer culture and serial treatment with activin A and bone morphogenetic protein (BMP). Where indicated, this differentiation protocol was modified by supplementation with ErbB agonists or antagonists. At 2 weeks following induction of differentiation with activin, cultures were enzymatically dispersed to single cells and replated on glass coverslips at low density. Unless otherwise stated, phenotyping studies were performed on days 20 to 25 postinduction, using cell preparations comprised of ~60% cardiomyocytes (Online Figure I). For selected experiments, hESC-CM cultures were transduced with a lentiviral vector in which the proximal promoter-enhancer region of the chicken GATA6 (cGATA6) gene drives expression of enhanced green fluorescent protein (EGFP). Parallel control experiments indicated ~50% transduction efficiency. Transduced cells were used in electrophysiological or immunocytochemical studies at 3 to 4 days after transduction, which corresponds to 22 to 25 days after induction with activin.

**Results**

**hESC-CMs Include Cardiomyocytes With Distinct Nodal and Working-Type AP Phenotypes**

We initially characterized the spontaneous AP properties of hESC-CMs resulting from our directed cardiac differentiation protocol. Because of the high cardiac purity of our preparation, we were able to patch-clamp cells in an unbiased fashion, rather than selecting cells with spontaneous contractile activity or a particular morphology. We used current-clamp techniques to record from a total of 61 cells, of which 12 were electrically nonexcitable (showing no spontaneous or stimulated APs) and 4 showed spontaneous AP characteristics deemed inconsistent with cardiomyocytes (ie, an AP duration to 90% repolarization [APD90] of <20 ms). The remaining 45 cells showed typical cardiac-type APs with distinct nodal- or working-type characteristics (Figure 1A and 1B).

To establish objective criteria for this classification, we analyzed histogram plots for a variety of parameters including spontaneous firing rate, APD90, upstroke velocity (dV/dtmax), AP amplitude (APA), and maximal diastolic potential (MDP). We identified a distinct population of hESC-CMs with a slow (dV/dtmax <15 V/sec) biventricular AP upstroke characteristic of nodal cardiomyocytes (Figure 1A and 1C). These putative nodal cells, which accounted for 31% (14 of 45) of recorded cardiomyocytes, consistently showed other expected AP properties, including a faster mean spontaneous firing rate, a narrower mean APA and a more depolarized mean MDP than the majority population of working-type cardiomyocytes (Table 1).

**Activation of the cGATA6-EGFP Transgene Identifies hESC-CMs With the Nodal Phenotype**

Although direct AP phenotyping is considered the “gold-standard” method of determining cardiac subtype, we also sought to develop a high-throughput, molecular approach that would be useful in screening effects following the manipulation of NRG-1β/ErbB and other signaling pathways. Because no validated genetic labels for early human nodal cells were available, we tested the hypothesis that the activation of a proximal promoter-enhancer element from the cGATA6 gene would specifically identify nodal-type hESC-CMs. Through elegant fate-mapping studies in transgenic mice, hESC-CMs expressing the EGFP reporter would be enriched by ~60%, allowing easy and rapid sorting of nodal cardiomyocytes from the remainder of the cardiac progeny.
because nodal cells are known to proliferate more slowly than their working-type counterparts in the developing heart.\textsuperscript{32,33} We pulsed transgenic day 25 hESC-CM cultures with the thymidine analogue bromodeoxyuridine for 24 hours and found that the cGATA6-EGFP\textsuperscript{+} cells had a significantly lower proliferative index than did the \(\beta\)-MHC\textsuperscript{+} hESC-CM population as a whole (Online Figure II, C).

We also confirmed that the cGATA6-EGFP\textsuperscript{+} cells had the expected nodal-type electrophysiological phenotype. Using criteria specified above, we found that 95\% (20 of 21) of EGFP\textsuperscript{+} cells showed nodal-type AP properties, versus only 10\% (2 of 20) of the EGFP\textsuperscript{−} cells (Figure 2C and 2D). (Note: only \(\approx\)50\% of hESC-CMs were transduced in these experiments, which may account for the EGFP\textsuperscript{−} cells with a nodal phenotype.) In addition to showing the characteristic slow, biphasic AP upstroke of nodal cells, the cGATA6-EGFP\textsuperscript{+} cells exhibited a more rapid mean spontaneous firing rate, a more depolarized mean MDP, and a narrower mean APA than their EGFP\textsuperscript{−} counterparts (Table 2; Online Figures III and IV [A]). Although a detailed comparison of all of the ionic conductances in cGATA6-EGFP\textsuperscript{+} and EGFP\textsuperscript{−} cells is beyond the scope of the present study, we used voltage-clamp techniques after Cho et al to survey the major currents in each group.\textsuperscript{34} In brief, after obtaining AP recordings, each cell was switched to voltage-clamp mode and was stepped from \(-40\) mV to a range of test potentials from \(-130\) mV to \(+60\) mV. Depolarizing steps elicited similar inward currents in EGFP\textsuperscript{+} and EGFP\textsuperscript{−} cells, but there was a different response to hyperpolarizing steps below \(-90\) mV (Online Figure IV, B through D). Although all hESC-CMs exhibited some degree of hyperpolarization-activated, time-dependent current (\(I_h\)), mean \(I_h\) current density was significantly larger in cGATA6-EGFP\textsuperscript{+} than in EGFP\textsuperscript{−} cells, again consistent with expectations for the nodal phenotype.\textsuperscript{31,34} Separate experiments confirmed that the peak \(I_h\) current was reduced by 62\%±5\% by ZD7288 (10 \(\mu\)mol/L), a blocker of hyperpolarization-activated and cyclic nucleotide–gated (HCN) channels (data not shown).

To test the function of this genetic label in human cells, we created a lentiviral vector in which the proximal cGATA6 promoter drives expression of EGFP and transduced hESC-CM cultures. By 48 hours post-transduction, approximately 15\% of cells were EGFP\textsuperscript{+}, and all of the EGFP\textsuperscript{+} cells immunostained positively for cardiac markers such as cardiac troponin T (Figure 2A), sarcomeric actin, and \(\beta\)-myosin heavy chain (\(\beta\)-MHC) (Online Figure II). Importantly, the cGATA6-EGFP\textsuperscript{+} cells showed uniformly high expression of the hyperpolarization-activated, pacemaker ion channel gene HCN4, which is perhaps the best validated and earliest expressed nodal cell marker (Figure 2B).\textsuperscript{35} We also examined the proliferation of the putative nodal cGATA6-EGFP\textsuperscript{+} cells, because nodal cells are known to proliferate more slowly than

![Figure 1. hESC-derived cardiomyocytes exhibit distinct nodal- and working-type action potentials.](image)

Table 1. Action Potential Parameters for Nodal and Working-Type hESC-CMs

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>(dV/dt_{\text{max}}) (V/sec)</th>
<th>Rate (bpm)</th>
<th>MDP (mV)</th>
<th>APA (mV)</th>
<th>APD(_{90}) (ms)</th>
<th>APD(_{95}) (ms)</th>
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<tr>
<td>Nodal CMs</td>
<td>14</td>
<td>6.5±0.9*</td>
<td>122.0±10.2*</td>
<td>(-47.2±1.2**)</td>
<td>74.9±3.2**</td>
<td>104.3±8.7*</td>
<td>178.2±11.6*</td>
</tr>
<tr>
<td>Working CMs</td>
<td>31</td>
<td>44.2±6.7</td>
<td>85.8±6.9</td>
<td>(-57.5±1.6)</td>
<td>96.8±2.7</td>
<td>144.7±15.7</td>
<td>211.1±17.2</td>
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APA indicates action potential amplitude; APD\(_{90}\) action potential duration measured at 50\% repolarization; APD\(_{95}\) action potential duration measured at 90\% repolarization; \(dV/dt_{\text{max}}\) maximum rate of action potential upstroke; MDP, maximum diastolic potential; N, cell no. *\(P<0.01\) and **\(P<0.05\) for nodal vs working cardiomyocytes.
pg/mL, respectively). In the developing and adult rodent heart, NRG-1β is produced by noncardiac cell types but signals through ErbB2 and ErbB4 receptors expressed by the cardiomyocytes themselves.15,16,35 Immunocytochemistry with antibodies against NRG-1/H9252, the ErbB receptors, and H9252-MHC suggests that this is also true in differentiating hESC-CM cultures (Figure 3B). Only rare NRG-1/H9252–expressing cells were observed (0.8±0.3% of the total cell population), and none of these cells expressed H9252-MHC. However, ErbB2 and ErbB4 receptors were expressed by all H9252-MHC–cardiomyocytes, but by very few (<5%) of the H9252-MHC–noncardiac cells. ErbB3 expression was observed in only a small fraction of the H9252-MHC–cardiac (0.9±0.2%) and total cell populations (4.0±0.2%).

Finally, we demonstrated functional NRG-1/H9252/ErbB signaling in hESC-CM cultures by analyzing 2 downstream effectors in this cascade, Akt/PKB and p42/p44 ERK. Both kinases were phosphorylated in response to treatment with NRG-1β and this response was inhibited in the presence of an anti–NRG-1/H9252 neutralizing antibody (Figure 3C).

Inhibition of NRG-1β/ErbB Signaling Enhances the Proportion of hESC-CMs With the Nodal Phenotype

To test the hypothesis that NRG-1β/ErbB signaling regulates the relative abundance of the 2 cardiac subtypes in differentiating hESC-CM cultures, we induced cardiac differentiation in the presence of either inhibitors or activators of NRG-1/H9252/ErbB signaling (Figure 4A). Interestingly, cultures treated with either anti–NRG-1β or the ErbB antagonist AG1478 commenced spontaneous beating earlier (days 9.0±0.6 and 10±0.6 postinduction, respectively) than did control or ex-

Table 2. Action Potential Parameters for cGATA6-EGFP+ and EGFP− hESC-CMs

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>dV/dt_{max} (V/sec)</th>
<th>Rate (bpm)</th>
<th>MDP (mV)</th>
<th>APA (mV)</th>
<th>APA_{50} (ms)</th>
<th>APA_{90} (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cGATA-EGFP+</td>
<td>21</td>
<td>10.5±1.2**</td>
<td>75.2±6.3*</td>
<td>52.1±1.5**</td>
<td>85.9±2.8**</td>
<td>114±11.5</td>
<td>212.9±24.3</td>
</tr>
<tr>
<td>cGATA-EGFP−</td>
<td>20</td>
<td>149.3±24.7</td>
<td>55.2±6.5</td>
<td>-64.1±1.5</td>
<td>106.5±1.5</td>
<td>171.0±26.3</td>
<td>278.6±31.1</td>
</tr>
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APA indicates action potential amplitude; APD_{50}, action potential duration measured at 50% repolarization; APD_{90}, action potential duration measured at 90% repolarization; dV/dt_{max}, maximum rate of action potential upstroke; MDP, maximum diastolic potential; N, cell no. *P<0.05 and **P<0.01 for cGATA6-EGFP+ vs EGFP− cardiomyocytes.
NRG-1/β-ErbB signaling changed the relative abundance of the 2 cardiac subtypes, as determined by both AP phenotyping and activation of the nodal-specific cGATA6-EGFP genetic reporter. To confirm these findings via an independent, molecular approach, we used quantitative RT-PCR to compare the expression of a panel of subtype-specific genes in control, AG1478- and NRG-1/β-tREATED hESC-CM cultures (Online Tables II and III). Because prior AP phenotyping studies had indicated a mixture of both cardiac subtypes under all 3 conditions, we were not expecting radical differences in the expression of subtype-specific genes in this experiment. Nonetheless, the changes in gene expression were remarkably consistent with our previous observations: 8 of 19 subtype-specific transcripts were found to be differentially expressed, and all 8 transcripts shifted in the hypothesized directions (Figure 5 and Online Table III). For example, AG1478-treated hESC-CMs showed 2.6-fold greater expression of Tbx-3 (TBX3) compared to control cells. Conversely, hESC-CMs treated with exogenous NRG-1/β showed 2.6-fold greater expression of the NPPA gene, which encodes for atrial natriuretic factor (ANF), a well-accepted marker of early chamber differentiation. Importantly, all 3 conditions showed similar expression of the pan-cardiac marker α-cardiac actin (ACTC1).

NRG-1/β-ErbB Signaling Regulates the Expression of Cardiac Subtype-Specific Genes

In the preceding experiments, manipulation of NRG-1/β-ErbB signaling changed the relative abundance of the 2 cardiac subtypes, as determined by both AP phenotyping and activation of the nodal-specific cGATA6-EGFP genetic reporter. To confirm these findings via an independent, molecular approach, we used quantitative RT-PCR to compare the expression of a panel of subtype-specific genes in control, AG1478- and NRG-1/β-treated hESC-CM cultures (Online Tables II and III). Because prior AP phenotyping studies had indicated a mixture of both cardiac subtypes under all 3 conditions, we were not expecting radical differences in the expression of subtype-specific genes in this experiment. Nonetheless, the changes in gene expression were remarkably consistent with our previous observations: 8 of 19 subtype-specific transcripts were found to be differentially expressed, and all 8 transcripts shifted in the hypothesized directions (Figure 5 and Online Table III). For example, AG1478-treated hESC-CMs showed 2.6-fold greater expression of the nodal-associated transcription factor Tbx-3 (TBX3) compared to control cells. Conversely, hESC-CMs treated with exogenous NRG-1/β showed 2.6-fold greater expression of the NPPA gene, which encodes for atrial natriuretic factor (ANF), a well-accepted marker of early chamber differentiation. Importantly, all 3 conditions showed similar expression of the pan-cardiac marker α-cardiac actin (ACTC1).

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To determine whether the effects of ErbB activation on subtype-specific genes were specific to NRG-1β/H9252, we treated hESC-CM cultures from day 5 to 25 with maximal concentrations of other ErbB agonists, including NRG-1α/H9251, epidermal growth factor (EGF), heparin-binding EGF-like growth factor and betacellulin (Online Figure VI). Consistent with its known effects on nonmesodermal ESC derivatives,39 betacellulin promoted the expansion of noncardiac cells, as indicated by a reduction in the expression of both pan- and subtype-specific cardiac genes and the absence of spontaneous beating activity in betacellulin-treated cultures. However, aside from the effect of betacellulin on overall cardiac purity, none of the ErbB agonists changed the expression of those working subtype-specific genes previously found to be enhanced by NRG-1β/H9252.

Manipulation of NRG-1β/ErbB Signaling Does Not Affect Cardiomyocyte Purity or Yield

One practical concern is that, although manipulation of NRG-1β/ErbB signaling might be useful in controlling cardiac subtype abundance in hESC-CM cultures, it could also mediate off-target effects and adversely affect overall cardiomyocyte purity and yield. To address this, we compared the percentage and yield of β-MHC+/cardiomyocytes in control, AG1478- and NRG-1β–treated hESC-CM cultures. Interestingly, no significant differences in cardiac purity or the yield of total cells or cardiomyocytes were observed, at least at 25 days postinduction (Figure 6A). We combined these measurements of cardiomyocyte yield with our AP phenotyping data (Figure 4B) to estimate the yield of each cardiac subtype under control and treated culture conditions. By this calculation, the control protocol yielded ~0.9 nodal and ~3.2 working myocytes per starting undifferentiated hESC. By contrast, AG1478 treatment produced ~1.7 nodal and ~1.5 working myocytes per starting undifferentiated hESC, versus ~0.3 nodal and ~4.1 working myocytes with exogenous NRG-1β.

Discussion

Multiple laboratories have reported that mESC- and hESC-CMs are electrophysiologically diverse and include both
working and nodal-type cardiomyocytes. This heterogeneity has been highlighted as a major challenge to the application of hESC-CMs to cardiac repair, because the transplantation of a significant population of myocytes with a sustained pacemaker phenotype could promote arrhythmias. Here, we report 2 complementary approaches that bring us closer to the goal of obtaining homogenous preparations of each cardiac subtype from hESCs. First, we have identified a genetic label that preferentially identifies nodal-type hESC-CMs. Although we originally developed the cGATA6-EGFP transgene to validate our AP phenotyping techniques and expedite the screening of candidate molecules, we anticipate the isolation of uniform populations via fluorescence-activated cell sorting in combination with this genetic label.

Second, we have demonstrated an endogenous signaling system that regulates cardiac subtype abundance in differentiating hESC-CM cultures (see model in Figure 6B). Contaminating noncardiac cell types release NRG-1/Erbb, which appears to favor working-type differentiation and likely contributes to the ∼80% of hESC-CMs that exhibit the working-type phenotype under control conditions. Treatment with additional exogenous NRG-1/β increased the expression of multiple working chamber-specific genes (Figure 5), and there was trend toward an increased percentage of working-type myocytes (Figure 4B). Conversely, when endogenous NRG-1/β/ErbB signaling was inhibited, there was a substantial increase in the proportion of hESC-CMs exhibiting the nodal phenotype, as indicated by 3 independent end points: AP phenotyping (Figure 4B), activation of the cGATA6-EGFP reporter (Figure 4C), and subtype-specific gene expression (Figure 5).

Phenotypic Properties of Nodal Versus Working-Type hESC-CMs

Working-type cardiomyocytes in the nascent atrial and ventricular chambers express markers including ANF and high-conductance gap junction proteins (connexins-40 and -43), and they exhibit greater proliferative activity and more rapid electric propagation than their nodal counterparts. Early nodal cells express markers including HCN4 and the transcription factors Tbx2 and Tbx3, and they are characterized by greater automaticity, lower proliferative activity, and slow propagation. In the present study, we have demonstrated distinct subsets of hESC-CMs that exhibit many of these phenotypic properties (summarized in Figure 6B).

One limitation of the present study is that the distinction between nodal and immature cardiomyocytes can be challenging, particularly in an in vitro system which lacks anatomic landmarks. Indeed, in the developing heart, nodal regions retain many of the phenotypic properties of the primitive heart tube heart, in part because the transcription factors Tbx2 and Tbx3 repress the chamber-specific gene expression program. That said, there is increasing molecular evidence that nodal myocytes do undergo some degree of specialization during development, which argues that a distinction can be made between nodal and merely primitive cardiomyocytes. Here, we present several pieces of evidence that inhibition of NRG-1/β/ErbB signaling is ex-
panding a population of true nodal hESC-CMs, rather than merely suppressing cardiomyocyte maturation. First, the AP phenotyping approaches used here have been used by multiple laboratories and are accepted, albeit low-throughput method of identifying cardiac subtype in ESC-CM cultures.4–9 Second, several of the genes responsive to NRG-1/β/ErbB manipulation are considered positive nodal markers that are specifically upregulated in nodal areas relative to primitive myocardium (eg, TBX3+). Third, NRG-1/β/ErbB-inhibited hESC-CM cultures actually commenced spontaneous beating earlier than their control and NRG-1/β-treated counterparts, an observation obviously inconsistent with a mere block of maturation. Our final piece of evidence comes from the use of the cGATA6-EGFP genetic label. In transgenic mice, the cGATA6 promoter used here does not show activity throughout the primitive myocardium, but instead undergoes preferential activation in regions of the cardiac crescent and heart tube fated to contribute to eventual nodal structures.29,42 Consistent with this, we observed EGFP expression in only a small minority of transduced hESC-CMs, and the nodal-like phenotype of these cells appears to be reasonably stable: in preliminary studies at 50 to 60 days postinduction, 8 of 10 cGATA6-EGFP+ hESC-CMs showed nodal-type AP properties comparable to those reported here at 25 days (data not shown). Also, cGATA6-EGFP+ hESC-CMs showed the low levels of proliferation expected of true nodal myocytes, whereas early hESC-CMs proliferate very rapidly.

Comparisons With Earlier Studies Examining the AP Phenotype of hESC-CMs

The AP parameters measured here for each hESC-CM subtype are in general agreement with previous reports.4–6,43,44 Most investigators concur that working-type outnumber nodal cells, although the precise ratio has varied slightly. Importantly, much of the published data are based on recordings from spontaneously beating clusters of hESC-CMs microdissected of embryoid body (EB) cultures. Although the latter approach could potential favor cells with greater automaticity, the high cardiac purity of our preparations enabled recording from cells in an unbiased fashion, rather than focusing on cells with greater spontaneity or a particular morphology.

Two other minor differences from earlier reports warrant discussion. First, many laboratories have reported a narrower range of values for dV/dtmax, ie, ~0 to 20 V/sec versus the 0 to 150 V/sec reported here. A likely explanation for this discrepancy is that most recordings have been obtained from clustered rather than individual hESC-CMs, as were used here. Indeed, Satin et al reported large dV/dtmax values similar to our own when recording from isolated hESC-CMs, and they noted that reductions in dV/dtmax would be expected in well-coupled multicellular preparations.43 It is also possible that hESC-CM maturation (and corresponding increases in fast sodium current density and dV/dtmax) proceed more rapidly via our directed differentiation protocol than under EB-based, high-serum culture conditions.

Another area of difference is that many investigators have further subdivided working-type ESC-CMs into atrial, ventricular, and even Purkinje fiber cardiomyocytes. The electrophysiological distinctions between these closely related subtypes are subtle at this state of maturation and were not obvious in our own AP dataset. Furthermore, many chamber-specific markers that are reliable in the adult heart (eg, ANF, myosin light chain-2a [MLC2a]17,38) are expressed in both atria and ventricles in the developing heart, so we lacked a convenient molecular marker to confirm any classification based on AP. Because this was a study where we needed straightforward, objective AP phenotyping criteria to screen for effects of NRG-1/β/ErbB signaling, we elected to focus instead on the unambiguous AP differences between nodal and working-type cells.

Comparisons With Earlier Studies Examining NRG-1/β/ErbB Signaling in Cardiac Development

NRG-1/β/ErbB signaling is thought to regulate 3 anatomically and temporally distinct steps in cardiac development: 1) early cardiogenesis,45–48 2) maturation and expansion of the primitive ventricle,14–16,24,25 and 3) induction of the peripheral conduction system.17,27 We interpret our findings in the hESC-CM system as consistent with the second function, ie, activation of NRG-1/β/ErbB signaling promotes the recruitment and/or expansion of early working-type hESC-CMs. Additional mechanistic studies are required, but we have unpublished data suggesting that NRG-1/β/ErbB signaling regulates differentiation into the working subtype, rather than differentially affecting the proliferation or survival of one subtype or another. Because hESC-CMs strongly express ErbB2 and ErbB4, we speculate that these effects are direct but cannot exclude the possibility of indirect signaling via ErbB activation in noncardiac intermediates.

Support for the role of NRG-1/β/ErbB signaling in early cardiogenesis comes from the Morisaki and Dai groups, both of whom observed increased cardiomyocyte yield from mESCs following NRG-1/β treatment.45–48 We did not observe any change in overall hESC-CM yield under conditions of altered NRG-1/β/ErbB signaling, but this may reflect the comparatively late window of treatment used in our experiments (>day 5 postinduction). Importantly, the aforementioned work with mESCs did not address the central question motivating the present study: whether NRG-1/β/ErbB regulates the ratio of nodal to working subtypes in already committed cardiomyocytes. Intriguingly, NRG-1/β treatment enhanced the expression of the chamber-specific marker MLC2a in differentiating mESC-derived cultures,55 but both groups otherwise relied on pan-cardiac markers or the activation of an Nkx2.5-EGFP transgene, a construct that is driven by a promoter with an uncertain relationship to nodal cardiomyocytes. (Recent reports indicate that a subset of nodal cells, including those contributing to the sinoatrial node, arise from Nkx2.5-null progenitors.)49

Our findings are not necessarily incompatible with prior work in the mouse model implicating NRG-1/β/ErbB signaling in the induction and patterning of the peripheral conduction (eg, Purkinje) system.17,27 The peripheral conduction system arises from committed working-type (specifically ventricular) cardiomyocytes, not from nodal progenitors50; and, in many respects, Purkinje cells represent an extreme of
the ventricular phenotype (ie, large cells with comparatively rapid conduction)\textsuperscript{51}. It is inviting to speculate that NRG-1/β treatment drives early “ventricularization” of hESC-CMs but might later induce these into Purkinje fibers (see model in Figure 6B). We presently lack suitably validated markers of early human Purkinje fibers to test this hypothesis.

On the other hand, our data do seem to contradict 2 prior studies in nonhuman models that suggested NRG-1/β treatment induces nodal differentiation. In the first of these 2 studies, Milan et al used antisense morpholino oligonucleotides to knock down NRG expression in zebrafish embryos.\textsuperscript{26} They then compared control and NRG-morphant hearts by calcium imaging and concluded that NRG was involved in the patterning of the slow-conducting nodal tissue of the AV ring. However, whereas conduction velocity in the AV node actually appeared little changed in the NRG-morphant hearts, propagation in the atrial and ventricular chambers was profoundly slowed (>5-fold). Put another way, the most striking phenotypic change following ablation of NRG-1/β/Erbb signaling was reduced functional maturation of the rapidly-conducting chamber myocardium, an observation consistent with our own findings in hESC-CM cultures. Subsequently, Ruhiparwar et al reported that NRG-1/β induced a “pacemaker-like” phenotype when applied to murine primary ventricular cardiomyocytes from the late fetal period.\textsuperscript{28} We attribute this apparent discrepancy to imprecision regarding the distinction between pacemaker (nodal) cells and myocytes of the Purkinje/peripheral conduction system, which, as noted above, have unique origins and phenotypic properties. We speculate that NRG-1/β actually induced Purkinje fiber differentiation in their study, and, in support of this, Ruhparwar et al reported that NRG-1/β treatment increased connexin-40 expression. Connexin-40 is a well-accepted early marker of cardiac chamber differentiation,\textsuperscript{52,53} so an increase in its expression would imply induction of working-type myocytes, not “pacemaker-like” cells as interpreted by the authors. However, connexin-40 expression later becomes restricted to the atria and peripheral conduction system,\textsuperscript{53} so it is entirely plausible that NRG-1/β promoted Purkinje fiber differentiation in their cultures.

Interestingly, Kim et al recently reported that genetically-selected hESC-CM preparations show less ventricular maturation than hESC-CMs in nonselected EB preparations of low cardiac purity.\textsuperscript{44} Our own findings raise the question of whether it is the removal of NRG-1/β—releasing noncardiac cells that underlies this effect. If so, highly purified hESC-CMs should be supplemented with NRG-1/β when mature working-type cardiomyocytes are desired.

Other Approaches to Controlling hESC-CM Heterogeneity

Results presented here demonstrate that NRG-1/β/Erbb signaling regulates cardiac subtype specification in differentiating hESC-CMs, but manipulation of this pathway is obviously not the only approach to reduce hESC-CM heterogeneity. As previously mentioned, work in model systems has implicated other factors in cardiac specialization (eg, endothelin,\textsuperscript{18} retinoic acid,\textsuperscript{19} and Wnts\textsuperscript{20}) so we are screening these for effects on hESC-CM cultures. Moreover, phenotypic changes have been observed in cardiomyocytes following culture on patterned 2D surfaces,\textsuperscript{54,55} within engineered 3D constructs,\textsuperscript{56,57} or under conditions of electric or mechanical stimulation.\textsuperscript{58} These culture conditions should be investigated for effects on hESC-CM heterogeneity. Finally, intracardiac grafting alters the phenotype of primary fetal ventricular cardiomyocytes,\textsuperscript{59} so it will be important to conduct transplantation experiments to determine how the complex signaling environment of the recipient heart\textsuperscript{60} affects the subtype and maturation of engrafted hESC-CMs.

Acknowledgments

We thank Benjamin Van Biber and Scott Lundy for technical assistance, as well as Drs Daniel Bowen-Pope, Kip Hauch, and Lil Pabon for critical reading of the manuscript and valuable comments.

Sources of Funding

This work was supported, in part, by a grant from Geron Corporation, as well as by NIH grants R01-HL064387, K08-HL80431, and P01-HL094374. M.A.L. has a sponsored research agreement and is a consultant with Geron. J.D.G. is an employee of Geron.

References


26. Milan DJ, Giokas AC, Serafica FC, Peterson RT, MacRae CA. Notch1 and neuregulin are required for specification of central cardiac conduction tissue. Development. 2006;133:1125–1132.


Novelty and Significance

What Is Known?

- Human embryonic stem cell–derived cardiomyocytes (hESC-CMs) are electrically diverse and exhibit nodal/pacemaker or working (atrial or ventricular chamber-like) electric phenotypes.
- A transgene containing a regulatory element from the chicken GATA6 (cGATA6) gene is preferentially activated in nodal regions of the developing and adult mouse heart.
- The neuregulin (NRG)-1/β/ErbB signaling pathway is involved in the development of specialized cardiac tissues, and neuregulin, ErbB2, and ErbB4 knockout mice all show a defect in early ventricular maturation.

What New Information Does This Article Contribute?

- Activation of the cGATA6 transgene preferentially identifies hESC-CMs with the nodal phenotype.
- Differentiating hESC-CM cultures exhibit an endogenous neuregulin/ErbB signaling system that regulates the expression of cardiac subtype-specific markers.
- Inhibition of the NRG-1/β/ErbB signaling pathway in differentiating hESC-CM cultures increases the proportion of myocytes with the nodal phenotype.

hESC-CMs have tremendous promise as a cell source for regenerative medicine, but existing preparations include admixed nodal and working-type myocytes. In this study, we demonstrate 2 complementary methods of deriving cardiac subtype-enriched preparations from hESCs: genetic selection based on the activation of the cGATA6-EGFP transgene and pharmacological manipulation of the NRG-1/β/ErbB signaling pathway. We first validated the cGATA6-EGFP genetic label by showing that it identifies a minority population of hESC-CMs with the immunophenotype and electrophysiological properties expected of nodal cells. Next, we tested the hypothesis that NRG-1/β/ErbB signaling regulates cardiac subtype abundance in differentiating hESC-CMs. Consistent with this hypothesis, we found that treatment with NRG-1/β/ErbB inhibitors greatly increased the proportion of nodal-type hESC-CMs, as indicated by action potential recordings, activation of the cGATA6-EGFP transgene, and subtype-specific gene expression. Conversely, despite the release of endogenous NRG-1/β by contaminating noncardiac cells, hESC-CM cultures treated with exogenous NRG-1/β nonetheless exhibited increased expression of multiple working chamber-specific genes and a trend toward an increased proportion of working-type myocytes. These findings provide important new insights into the development of specialized cardiac subtypes in early stages of human heart formation, and they suggest practical approaches to derive homogeneous populations of cardiomyocytes for cell therapy and in vitro modeling.
Neuregulin/ErbB Signaling Regulates Cardiac Subtype Specification in Differentiating Human Embryonic Stem Cells
Wei-Zhong Zhu, Yiheng Xie, Kara White Moyes, Joseph D. Gold, Bardia Askari and Michael A. Laflamme

_Circ Res._ 2010;107:776-786; originally published online July 29, 2010;
doi: 10.1161/CIRCRESAHA.110.223917

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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SUPPLEMENTAL MATERIAL

Detailed Methods

hESC Culture and Cardiac Differentiation

H7 hESCs were maintained in the undifferentiated state on Matrigel (BD Biosciences) coated plates in mouse embryonic fibroblast conditioned medium (MEF-CM) supplemented with 4 ng/ml bFGF (Peprotech), as previously described1. For all experiments, hESCs were differentiated into cardiomyocytes using our recently reported directed cardiac differentiation protocol2. (Please see Figure 4A.) In brief, undifferentiated hESCs were detached by a 10-minute incubation with 0.5 mM EDTA, followed by replating onto Matrigel-coated surfaces in a high-density monolayer culture (4x10^4 hESCs per well of a 96-well plate) and maintenance for an additional 6 days in MEF-CM plus bFGF. To induce cardiac differentiation, the medium was then changed to RPMI-1640 and B27 supplement (Invitrogen) plus growth factors: 100 ng/ml human recombinant activin A (R&D Systems) for 24 hours, followed by 10 ng/ml human recombinant bone morphogenetic protein-4 (BMP-4, R&D Systems) for 4 days. Thereafter, the medium was exchanged for RPMI-B27 without supplementary growth factors every other day. Cardiogenesis was indicated by the appearance of widespread spontaneous beating activity ~12 days following induction with activin A.

After replating for electrophysiological studies as described below, the final cardiomyocyte purity was 59 ± 6%, based on immunostaining with an antibody against sarcomeric actins (Online Figure I). Similar estimates of cardiac purity were obtained using antibodies against cardiac troponin T (Online Figure I) and β-myosin heavy chain (Figure 6A). In terms of non-cardiac cells, CD31+ endothelial cells comprised <1% of the total cell population (Online Figure I), while no cytokeratin+ epithelial cells or α-fetoprotein+ endodermal derivatives were present.

Where indicated, differentiating hESC-CM cultures were further supplemented with activators or inhibitors of ErbB signaling either from days 5-12 or continuously after day 5 post-induction of differentiation with activin. The day 5-12 treatment window was selected based on pilot RT-PCR studies indicating the initial up-regulation of subtype-specific genes occurred during this period (data not shown). Reagents employed included neuregulin (heregulin)-1α (100 ng/mL, R&D Systems), neuregulin-1β (100 ng/mL, R&D Systems), anti-neuregulin-1β neutralizing antibody (25 μg/ml, R&D Systems), AG1478 (10 μmol/L, Calbiochem), epidermal growth factor (EGF, 100 ng/mL, Prospec), heparin-binding EGF-like growth factor (HB-EGF, 100 ng/mL, Peprotech) and betacellulin (100 ng/mL, Prospec). Where indicated, control cultures were treated with either non-immune IgG (25 μg/ml, R&D Systems) or DMSO (0.1%, Sigma-Aldrich) vehicle.

Generation and use of the cGATA6-EGFP lentiviral vector

The pPD46.21 plasmid containing the proximal (-1.5/+0.0) promoter-enhancer region of the chicken GATA6 (cGATA6) gene was generously provided by Dr. John Burch (Fox Chase Cancer Center)3. To generate the cGATA6-EGFP lentiviral vector, the 1.5 kb cGATA6
promoter-enhancer region was excised from the pPD46.21 plasmid by digestion with the restriction enzymes Sal I and Age I. This promoter-enhancer fragment was ligated into the lentiviral transfer plasmid pJGL2-EGFP (generously provided by Drs. Jonathan Golob and Charles Murry, University of Washington), which includes a transgene in which EGFP expression is driven by the constitutive elongation factor-1α (EF1α) promoter, a central polyurine tract, and a woodchuck hepatitis virus post-transcriptional regulatory element4. For this, the EF1α promoter DNA was excised from pJGL2-eGFP plasmid, also using Sal I and Age I digestion, followed by replacement with the cGATA6 promoter-enhancer fragment.

VSV-G-pseudotyped lentiviral particles were generated and concentrated as previously described4, 5. In brief, 6x10^6 HEK293D cells seeded on a 15 cm^2 plate 24 hours prior to co-transfection with the following plasmids: 8 µg of envelope plasmid pMK-VSVG, 15 µg of pMDL-G/P-RPE plasmid expressing the HIV-1 gap/pol and tat genes, 11.5 µg of pRSV-REV plasmid expressing the HIV-1 rev protein, and 29 µg of either the cGATA6-EGFP or EF1α-EGFP lentiviral transfer vector construct. Supernatant containing the resultant viral particles was collected at 72 hours following transfection, concentrated by filtration (Millipore Centricon Plus-20 columns with a molecular weight cutoff of 10kD), and stored at -80°C. Lentiviral stocks were titered by viral p24gag ELISA (QuickTiter Lentiviral Quantitation kit, Cell Biolabs).

Prior to lentiviral transduction, hESC-CMs were replated onto glass coverslips as described below for electrophysiological studies and allowed to recover for 4-5 days. Cells were then exposed to cGATA6-EGFP lentivirus (at 5000 LPs/cell, added to their usual RPMI-B27 medium) for 12 hours. Parallel transduction with an equivalent quantity of the constitutively-expressing EF1α-EGFP lentiviral vector was routinely performed and indicated that this viral titer results in the reliable transduction of ∼50% of target hESC-CMs. Transduced cell preparations were then used in either electrophysiological or immunocytochemical studies at 3-4 days post-transduction. (In an unsuccessful attempt to derive homogenous preparations of transduced cells, we generated a modified, bicistronic cGATA6-EGFP lentiviral vector that also included a selection cassette in which the phosphoglycerate kinase promoter drives expression of neomycin resistance. However, we found that this second cassette interfered with the specificity and function of the cGATA6-EGFP transgene (data not shown)).

Patch-clamp studies

At 14 days following induction with activin A, hESC-CMs were dispersed using 0.1 U/ml of dispase (Invitrogen) with 63 U/ml DNase I (Invitrogen) and replated at low density onto 0.5% gelatin-coated glass coverslips. After a few days of additional culture, the spontaneously generated action potentials (APs) of the hESC-CMs were recorded using a HEKA EPC-10 amplifier (HEKA, Lambrecht, Germany), operated in current-clamp mode. (Note that the EPC-10 has a true “voltage follower” circuit, similar to a classical microelectrode amplifier6, 7). After obtaining gigaohm seal, electrical access to the cells was obtained via the β-escin perforated patch technique6, 9, which we found improved success relative to the conventional ruptured patch approach, as it greatly minimized seal disruption. Patch pipettes with a resistance of 2-4 MΩ were used; cells with a series resistance of >10 MΩ were discarded. The
capacitance of the examined cells was 17.5 ± 7.6 pF (range 5.8-32.8 pF), in comparison to the ~150 pF typically reported for adult human ventricular myocytes. All recordings were performed at 36 ± 1 °C, using the following bath medium: (in mM) 140 NaCl, 5.4 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 0.33 NaH₂P0₄, 5 dextrose, and 10 HEPES, adjusted to pH 7.40 with NaOH. The pipette solution was (in mM) 135 KCl, 5 Na₂ creatine phosphate, 5 MgATP and 10 HEPES, adjusted to pH 7.20 with KOH. Data were digitized at 10 Hz and filtered at 2.9 Hz. Action potential parameters were analyzed by an individual blinded to culture conditions, using Patchmaster (HEKA) and IgorPro software.

The voltage-clamp studies comparing cGATA6-EGFP and EGFP- cells (depicted in Online Figure III) were modeled after a study by Cho et al, which analyzed membrane currents in primary murine sinoatrial myocytes. For this work, we minimized the series resistance error by using the conventional ruptured patch technique and ~50-70% compensation. After obtaining spontaneous action potential recordings as previously described, each hESC-CM was switched to voltage-clamp mode (and a -40 mV hold) with no change in ionic conditions. The cell was then cycled at 0.5 Hz through a series of test potentials ranging from -130 mV to +60 mV at 10 mV intervals; each test potential lasted 1 second in duration. The magnitude of the resultant initial membrane current was then measured either at the peak of the inward current (in the case of depolarizing test potentials) or 5 milliseconds after the step (hyperpolarizing test potentials). The magnitude of the late membrane current was measured by averaging the current amplitude over the final 15 milliseconds of the test pulse. The hyperpolarization-activated, time-dependent current (Iₜₜ) was then expressed as the difference between the initial and late membrane current amplitudes. Note that we also attempted to quantitate the Iₜₜ tail current upon return to the hold potential, but we found that this tail current often overlapped with a large inward fast sodium current (INa) that could not be satisfactorily blocked by tetrodotoxin or other maneuvers in all cells.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared by lysing cell preparations with the Qiagen RNeasy kit, followed by DNase treatment. After confirming the quality of the RNA using an Agilent Bioanalyzer 2100, it was reverse-transcribed into cDNA using the Superscript III first-strand cDNA synthesis kit (Invitrogen). Online Tables I and II list the primers used for semi-quantitative and quantitative RT-PCR reactions, respectively. All primer pairs were designed to be intron-spanning. Quantitative real-time PCR reactions were performed using the SYBR green dye system and an Applied Biosystems 7900HT instrument. Cycling conditions were 10 minutes at 95 °C, and 40 cycles of 30 seconds at 95 °C, 30 seconds at 58 °C, and 30 seconds at 72 °C. mRNA levels were normalized using GAPDH as an internal control, and adult human heart cDNA was always run in parallel as a positive control.

Western blot analysis of Akt and Erk phosphorylation

To demonstrate functional NRG-1β/ErbB signaling in hESC-CM cultures, we used Western blotting to analyze two proximal effectors in this signal transduction cascade, the Akt and Erk kinases. For this, hESC-CMs (~5x 10⁶ million per well) were treated on day 10.
post-induction with 0, 10, or 100 ng/ml NRG-1β (R&D Systems) in the presence or absence of 25 μg/ml anti-NRG-1β neutralizing antibody (R&D Systems) for 10 minutes. After treatment, cells were rinsed twice with ice-cold PBS and lysed (on ice) with 400 μl of extraction buffer (25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 10 mM Na₂SO₄, 50 mM NaF and 1% Triton X-100 supplemented just prior to use with 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Sigma)). Lysates were collected with a cell scraper, placed in microcentrifuge tubes, vortexed for 3 minutes at 4 ºC, and then spun at 4 ºC for 20 minutes at 12,000 g to eliminate cell debris. The resultant lysates were denatured at 95 ºC for 10 minutes, loaded (at 30 μg/lane) onto a 10% SDS/polyacrylamide gel, electrophoresed, and transferred onto a PVDF membrane. The membrane was blocked for 1 hour at room temperature in 5% nonfat-milk, dissolved in 1X TBST (150 mM NaCl, 10 mM Tris-HCl (pH 7.4), and 0.1% Tween), incubated overnight with total and anti-phospho Akt/PKB and anti- p42/44 phospho-ERK antibodies (Cell Signaling), and then additionally incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Sigma). The resultant bands were visualized using an enhanced chemiluminescent detection kit (Amersham).

Immunocytochemistry

Dissociated hESC-CMs were cultured on glass cover slips, fixed, and immunostained as previously described 2, 12-14. See Online Table IV for detailed information regarding the source, fixation conditions, and titer for each of the primary antibodies employed in this study, as well as the positive control tissue used to confirm antibody specificity. (Because others have previously reported particular problems with the specificity of commercially-available, isoform-specific anti-ErbB antibodies, we rigorously confirmed that each used here had the appropriate pattern of immunoreactivity at different skeletal muscle locations 15.) Unless otherwise stated, detection was performed using Alexa-488 or -594 conjugated secondary antibodies (Molecular Probes), followed by counterstaining with Hoechst 33342.

Immunocytochemical detection of bromodeoxyuridine (BrdU)-labeled cells was performed somewhat differently, using a protocol that has also been previously described 12. In brief, cell populations were first immunostained with anti-β-MHC or EGFP primary antibodies, followed by detection with an appropriate species-specific biotinylated goat secondary antibody, avidin-biotinylated alkaline phosphatase complex (Vector ABC-AP), and the chromogenic substrate Vector Red. Next, BrdU immunostaining was performing by antigen retrieval (HCl and Borax Buffer), detection with a peroxidase-conjugated anti-BrdU antibody (1:50, Roche) and 3,3’-diaminobenzidine (DAB, brown) development. Hematoxylin was then used as a nuclear counterstain.

All cell counts were performed by an observer blinded as to the preceding treatment conditions. Each experimental condition was assayed in triplicate, and a minimum of 500 nuclei were counted per sample.
ELISA

Supernatant was collected from day 0 hESC and day 10 post-differentiation hESC-CM cultures and stored at -80° C. The concentration of soluble NRG-1β was then determined using a commercially-available ELISA kit (Raybiotech, Norcross, GA), as per vendor instructions.

Statistics

When analyzing the proportion of hESC-CMs exhibiting each action potential phenotype under various treatment conditions, groups were compared using Fisher’s exact test with Bonferroni correction (with $\alpha=0.05$ for significance). In the statistical analysis of all other experiments, we used ANOVA followed by post hoc Student’s t-testing with Bonferroni correction. Values are expressed as means ± standard error.
Online Figure I. Composition of directly-differentiated hESC-CM cultures. Cardiac differentiation was induced in hESC cultures by serial treatment with activin A and BMP-4 (see Figure 4A). The resultant differentiated cells were re-plated and prepared for electrophysiological studies as described elsewhere. On day 25 post-induction with activin, these preparations were found to be 59 ± 6% positive for the striated muscle marker sarcomeric actins (sActin) by immunocytochemistry, and a similar percentage was immunoreactive for cardiac troponin T (cTnT). Most of the non-cardiac cells showed a fibroblastic morphology and could not be identified by immunostaining. CD31+ endothelial cells (arrow) were rare (<1% of the total cell population), and no pan-cytokeratin+ epithelial cells or α-fetoprotein+ endodermal derivatives were identified. Scalebar in all images = 50 μm.
Online Figure II

A

B

C

EGFP  BrdU

EGFP

β-MHC

% of BrdU+ Cells

Day 15  Day 25  Day 50  Day 25

β-MHC+ hESC-CMs  cGATA6+ hESC-CMs

S7
Online Figure II. Cardiac immunophenotype and proliferative index of cGATA6-EGFP positive cells.  

A: Representative cGATA6-EGFP+ cell dual-immunostained with antibodies against EGFP and the striated muscle marker sarcomeric actin (sActin).  

B: The differential interference contrast (DIC) image to the left includes both cGATA6-EGFP+ and EGFP- cells. As illustrated by the corresponding field to the right, all of the cGATA6-EGFP+ cells also expressed the cardiac marker β-myosin heavy chain.  

C: Transgenic cGATA6-EGFP hESC-CM cultures were pulsed with the thymidine analogue bromodeoxyuridine (BrdU) for 24 hours at various time points, and the fraction of EGFP+ or total β-MHC+ cardiomyocytes with incorporated label was determined by immunocytochemistry. The photomicrograph to the left shows a representative field of day 25 cGATA6-EGFP hESC-CMs that have been dual-immunostained with anti-EGFP (red) or anti-BrdU (brown) antibodies. As illustrated by the graph to the right, the percentage of BrdU-labeled β-MHC+ hESC-CMs declined with duration in culture, but the cGATA6-EGFP+ cells showed a lower proliferative index than the equivalently aged hESC-CM population as a whole. No such differences in BrdU labeling were observed when comparing wildtype and hESC-CMs transduced with control EF1α-EGFP lentivirus (data not shown). N=3 biological replicates; * p<0.05. Scalebar in all images = 20 μm.
Online Figure III. Action potential properties of cGATA6-EGFP positive and negative hESC-CMs. Histogram plots superimposing the number of cGATA6-EGFP+ (green bars) and EGFP- cardiomyocytes (black hatched bars) exhibiting any given value for the following spontaneous action potential parameters: maximal upstroke velocity (dV/dt max, A), rate (B), maximum diastolic potential (MDP, C), maximum action potential amplitude (APA, D), action potential duration at 50% repolarization (APD 50, E), and action potential duration at 90% repolarization (APD 90, F). Recall that, because only ~50% of cells were transduced, we expected the cGATA6-EGFP-null population to include both putative working-type and non-transduced hESC-CMs. Despite this heterogeneity in the EGFP- population, the cGATA6-EGFP+ cells typically exhibited a slower dV/dt max, a more depolarized MDP, and a narrower APA than their EGFP- counterparts, findings consistent with the hypothesis that activation of the cGATA6-EGFP reporter identifies nodal-type hESC-CMs. N= 21 EGFP+ and 20 EGFP- cells.
Online Figure IV

A

B

C

D

EGFP^+  EGFP^-

+30 mV  0 mV  -30 mV

-60 mV  -90 mV  -120 mV

-100  +50 mV

-10 pA/pF

h Current Density at -120 mV (pA/pF)

EGFP^-  EGFP^+

S11
Online Figure IV. Differences in action potentials and whole-cell membrane currents between cGATA6-EGFP positive and negative hESC-CMs.  

A: Spontaneous action potentials were recorded from representative cGATA6-EGFP+ and EGFP- hESC-CMs (left and right upper traces, respectively). The corresponding lower traces plot dV/dt versus time for these same cells. Note, among other differences in action potential properties (e.g. rate, maximum diastolic potential, and plateau phase waveform), the EGFP+ cell showed a substantially slower and more complex action potential upstroke phase than did its EGFP- counterpart.  

B: After completing action potential recordings, each cell was switched to voltage-clamp mode and stepped at 0.5 Hz from a holding potential of -40 mV to a range of test potentials from -130 mV to +60 mV (1 second duration). The resultant membrane currents for each cell (cGATA6-EGFP+: green, EGFP-: black) were then measured at the beginning (O) and end (●) of each voltage step. Note that strongly hyperpolarizing pulses elicited a time-dependent, slowly-activating inward current that was substantially larger in the EGFP+ cell.  

C: Plot depicting the full current-voltage (I-V) relationship for the initial (O) and late (●) membrane currents, elicited in each cell as described for panel B.  

D: Mean amplitude of the hyperpolarization-activated, time-dependent current (I_h) in cGATA6-EGFP+ and EGFP- hESC-CMs, measured as the difference between initial and late current densities during a step to -120 mV. N= 10 EGFP+ and 9 EGFP- cells.  

* p<0.05.
Online Figure V. **Differentiating hESC-CM cultures treated with NRG-1/ErbB inhibitors show reduced cardiomyocyte proliferation.**

**A:** Differentiating hESC-CM cultures were treated with vehicle, anti-NRG-1β, AG1478, or exogenous NRG-1β as indicated. On day 24 post-induction with activin A, control and treated cultures were pulsed with BrdU for 24 hours and then fixed for immunocytochemistry.

**B:** The percentage of β-MHC+ hESC-CM with incorporated BrdU under each condition, as determined by dual immunohistochemistry. Note that hESC-CMs differentiated under conditions of chronic NRG-1β/ErbB inhibition showed a significantly lower proliferative index. Note also that this observation cannot be attributed to the inhibition of a direct mitogenic effect by endogenous NRG-1β, because the anti-NRG-1β neutralizing antibody was not present during the BrdU pulse.
Online Figure VI. Treatment with other ErbB agonists does not mediate the effects on cardiac subtype-specific gene expression observed with NRG-1β. Differentiating hESC-CM cultures were treated from days 5-25 post-differentiation with ErbB agonists including epidermal growth factor (EGF, red), heparin-binding EGF (HB-EGF, yellow), betacellulin (BTC, blue), and NRG-1α (brown). The legend indicates the ErbB receptor isoform(s) preferred by each of these agonists. Control (white) and treated cultures were then analyzed using quantitative RT-PCR for those subtype-specific genes that had been previously shown to be altered by NRG-1β signaling (see main Figure 5). No statistically significant effects in gene expression were observed, except in the case of BTC. Rather than mediating a cardiac subtype-specific effect, BTC appeared to greatly promote the expansion of non-cardiac cell types, as evidenced by a significant reduction in the expression of the pan-cardiac gene ACTC1 (α-cardiac actin) and the absence of spontaneous beating activity in BTC-treated cultures. * p<0.05, ** p<0.01 vs. control group.
Online Table I.  Primer sets used for semi-quantitative RT-PCR.

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## Online Table II. Primer sets used for quantitative RT-PCR analysis.

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<td>α-cardiac actin</td>
<td>NM 005159</td>
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<td>TGTCTCCCCTCCATGCTCAGT</td>
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<tr>
<td>CACNA1C</td>
<td>Cav1.2 (I_CaL)</td>
<td>NM 000719</td>
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<td>GCTTCACAAAGAGGCTGTG</td>
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<td>CANCA1G</td>
<td>Cav3.1 (I_CaT)</td>
<td>NM 198397</td>
<td>CTTCACACCCTATGCTGCTC</td>
<td>CTGCTCCACCATGCTAGCCT</td>
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<td>GJA1</td>
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<td>NM 000165</td>
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<td>TGAAGCTGAACATGACCCGA</td>
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<td>GJA5</td>
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<td>NM 181703</td>
<td>GCAGCCTAGCTITCAAAATG</td>
<td>GTGACAGATGTGGGAGGAGAT</td>
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<td>GJA7</td>
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<td>NM 005497</td>
<td>TCTCCTTGGCTTCCCTTATG</td>
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<td>HCN1</td>
<td>(I&lt;sub&gt;i&lt;/sub&gt;/ I&lt;sub&gt;h&lt;/sub&gt;)</td>
<td>NM 021072</td>
<td>GTGACAGAAGCAGGGTTAAT</td>
<td>ATTGCCAGTTGAGAGATCA</td>
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<td>HCN2</td>
<td>(I&lt;sub&gt;i&lt;/sub&gt;/ I&lt;sub&gt;h&lt;/sub&gt;)</td>
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<td>TCTCCTTGGCTTCCCTTATG</td>
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<td>HCN4</td>
<td>(I&lt;sub&gt;i&lt;/sub&gt;/ I&lt;sub&gt;h&lt;/sub&gt;)</td>
<td>NM 005477</td>
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<td>GTTGGAGCCACTGATGAG</td>
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<td>KCNE1</td>
<td>mink, ISK (I&lt;sub&gt;Ks&lt;/sub&gt;)</td>
<td>NM 000219</td>
<td>CAGGCCCAGATTACAGGAGA</td>
<td>GCAGAACTGCTGTGCTGTT</td>
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<td>KCNE2</td>
<td>MiRP1 (I&lt;sub&gt;Kr&lt;/sub&gt;, I&lt;sub&gt;i&lt;/sub&gt;, I&lt;sub&gt;o&lt;/sub&gt;)</td>
<td>NM 172201</td>
<td>TCATGGTGATGATGGAAATTG</td>
<td>TTATCAGGGGAGCTTTGA</td>
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<td>KCNJ2</td>
<td>Kir2.1 (I&lt;sub&gt;K1&lt;/sub&gt;)</td>
<td>NM 000891</td>
<td>TTGTCAAGAGCCAAACCAACA</td>
<td>AGCAACACACATGTGGGAAT</td>
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<td>MYL2</td>
<td>MLC-2v</td>
<td>NM 000432</td>
<td>CGTTCCGGGAATGCTGACACCACGC</td>
<td>AGTCCAAAGTCCACTGACGAG</td>
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<td>MYL7</td>
<td>MLC-2a</td>
<td>AK311869</td>
<td>TCAAGGCTTACGACAGAATCG</td>
<td>AAGACGGAATTTGATGGG</td>
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<tr>
<td>NKX2-5</td>
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<td>NM 004387</td>
<td>CCCCTGGATTTTGCATTCAT</td>
<td>GGGAGCAGTAAAGACACAG</td>
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<td>NPPA</td>
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<td>NM 006172</td>
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<td>GCCTGAGCTAGGACCTGAAAA</td>
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<td>SCN5A</td>
<td>Nav1.5 (I&lt;sub&gt;Na&lt;/sub&gt;)</td>
<td>NM 000335</td>
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<td>AGGACTCAACTGGCTTCTT</td>
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<td>TBX2</td>
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<td>NM 005994</td>
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<td>GCATGGATGATGGATGGTG</td>
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<tr>
<td>TBX3</td>
<td></td>
<td>NM 016569</td>
<td>ATTTTCACAATCTCGGTGAG</td>
<td>TATAATCTCCCCCTGCCACGT</td>
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<td>TBX5</td>
<td></td>
<td>NM 080718</td>
<td>TCCAGAAAACACTCGACACC</td>
<td>TGGCAAGGGATTATTCTCA</td>
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</table>
Online Table III. Quantitative RT-PCR analysis of cardiac subtype-specific gene expression in control, NRG-, and AG1478-hESC-CMs.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold-Change Relative to Control hESC-CMs</th>
<th>Gene expression pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NRG-1β hESC-CMs</td>
<td>AG1478 hESC-CMs</td>
</tr>
<tr>
<td>ACTC1</td>
<td>1.11 ± 0.13</td>
<td>1.05 ± 0.03</td>
</tr>
<tr>
<td>CACNA1C</td>
<td>0.39 ± 0.07*</td>
<td>0.91 ± 0.17##</td>
</tr>
<tr>
<td>CANCA1G</td>
<td>0.25 ± 0.04*</td>
<td>1.47 ± 0.24##</td>
</tr>
<tr>
<td>GJA1</td>
<td>1.57 ± 0.20*</td>
<td>1.00 ± 0.09##</td>
</tr>
<tr>
<td>GJA5</td>
<td>1.13 ± 0.19</td>
<td>0.76 ± 0.25</td>
</tr>
<tr>
<td>GJA7</td>
<td>1.02 ± 0.41</td>
<td>1.06 ± 0.15</td>
</tr>
<tr>
<td>HCN1</td>
<td>0.72 ± 0.27</td>
<td>1.54 ± 0.69</td>
</tr>
<tr>
<td>HCN2</td>
<td>0.76 ± 0.10</td>
<td>1.04 ± 0.32</td>
</tr>
<tr>
<td>HCN4</td>
<td>0.64 ± 0.17</td>
<td>1.96 ± 0.41*##</td>
</tr>
<tr>
<td>KCNE1</td>
<td>1.04 ± 0.20</td>
<td>1.03 ± 0.37</td>
</tr>
<tr>
<td>KCNE2</td>
<td>1.56 ± 0.98</td>
<td>2.43 ± 2.02</td>
</tr>
<tr>
<td>KCNJ2</td>
<td>1.05 ± 0.42</td>
<td>0.37 ± 0.18</td>
</tr>
<tr>
<td>MYL2</td>
<td>0.81 ± 0.38</td>
<td>0.13 ± 0.05**#</td>
</tr>
<tr>
<td>MYL7</td>
<td>0.69 ± 0.32</td>
<td>1.01 ± 0.42</td>
</tr>
<tr>
<td>NNX2.5</td>
<td>1.06 ± 0.43</td>
<td>1.39 ± 0.78</td>
</tr>
<tr>
<td>NPPA</td>
<td>2.56 ± 0.80*</td>
<td>1.22 ± 0.11##</td>
</tr>
<tr>
<td>SCN5A</td>
<td>1.41 ± 0.24</td>
<td>0.65 ± 0.22##</td>
</tr>
<tr>
<td>TBX2</td>
<td>0.77 ± 0.17</td>
<td>1.58 ± 0.49</td>
</tr>
<tr>
<td>TBX3</td>
<td>1.64 ± 1.15</td>
<td>2.63 ± 0.92**</td>
</tr>
<tr>
<td>TBX5</td>
<td>0.69 ± 0.14</td>
<td>2.14 ± 0.98</td>
</tr>
</tbody>
</table>

Gene expression in NRG-1β-treated hESC-CMs, AG1478-treated hESC-CMs, and adult human heart, normalized to expression in control (untreated) hESC-CM cultures. The primers used in this study are listed above in Online Table II. Values are mean ± SE (from 4 biological replicates). Expression in hESC-CM groups were compared by Bonferroni corrected one-way ANOVA with *P < 0.05 vs. control, **P < 0.01 vs. control, †P < 0.05 vs. NRG-treated, and ‡P < 0.01 vs. NRG-treated. Genes that showed at least one statistically significant difference between groups are highlighted and also appear in Figure 5 of the main text. The right-most column lists the expected pattern of expression for each gene, as well as supporting references.
Online Table IV.  Primary antibodies employed.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody Type (Clone Name or Catalog #)</th>
<th>Vendor</th>
<th>Fixative</th>
<th>Titer</th>
<th>Positive Control(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-fetoprotein</td>
<td>Rabbit polyclonal Ab2413</td>
<td>Abcam</td>
<td>PF</td>
<td>1:100</td>
<td>Rat fetal liver</td>
</tr>
<tr>
<td>α-sarcomeric actin</td>
<td>Mouse monoclonal (Clone 5C5)</td>
<td>Sigma-Aldrich</td>
<td>PF</td>
<td>1:2500</td>
<td>Rat and human myocardium</td>
</tr>
<tr>
<td>β-MHC</td>
<td>Mouse monoclonal (Clone A4.951)</td>
<td>ATCC</td>
<td>MeOH</td>
<td>1:10</td>
<td>Human and guinea pig myocardium</td>
</tr>
<tr>
<td>Cardiac Troponin T</td>
<td>Mouse monoclonal (Clone 13-11)</td>
<td>Thermo Scientific</td>
<td>PF</td>
<td>1:100</td>
<td>Human and guinea pig myocardium</td>
</tr>
<tr>
<td>CD31/PECAM</td>
<td>Mouse monoclonal (Clone JC70A)</td>
<td>Dako</td>
<td>PF</td>
<td>1:100</td>
<td>Cultured human endothelial cells</td>
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<tr>
<td>ErbB2</td>
<td>Rabbit polyclonal (C-18)</td>
<td>Santa Cruz</td>
<td>Ace</td>
<td>1:100</td>
<td>Rat soleus and EDL skeletal muscles</td>
</tr>
<tr>
<td>ErbB3</td>
<td>Mouse monoclonal (Clone RTj.1)</td>
<td>BD</td>
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<td>1:100</td>
<td>Rat EDL skeletal muscle</td>
</tr>
<tr>
<td>ErbB4</td>
<td>Rabbit polyclonal (C-18)</td>
<td>Santa Cruz</td>
<td>Ace</td>
<td>1:100</td>
<td>Rat soleus and EDL skeletal muscles</td>
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<tr>
<td>GFP</td>
<td>Rabbit polyclonal (ab6556)</td>
<td>Abcam</td>
<td>PF</td>
<td>1:1000</td>
<td>EGFP+ transgenic mouse</td>
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<tr>
<td>HCN4</td>
<td>Mouse monoclonal (Clone N114/10)</td>
<td>UC Davis/ NeuroMab</td>
<td>MeOH</td>
<td>1:100</td>
<td>Porcine sinoatrial node</td>
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<tr>
<td>Neuregulin-1β</td>
<td>Mouse monoclonal (7D5)</td>
<td>NeoMarkers</td>
<td>MeOH</td>
<td>1:250</td>
<td>Rat SOL and EDL skeletal muscles</td>
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<tr>
<td>Pan-cytokeratins (epithelium)</td>
<td>Mouse monoclonal (Clone AE1/AE3)</td>
<td>Dako</td>
<td>PF</td>
<td>1:100</td>
<td>Human epidermis</td>
</tr>
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</table>

Abbreviations are as follows: Ace= acetone, ATCC= American Type Culture Collection, β-MHC= β-myosin heavy chain, EGFP= enhanced green fluorescent protein, EDL = extensor digitorum longus, MeOH= methanol, PF= paraformaldehyde.
Online Supplemental References


with podoplanin expression in cells from the developing cardiac pacemaking and conduction system. *Anat Rec (Hoboken).* 2007;290:115-122.


